FEBS Letters 580 (2006) 6603–6611

Methylation of Smad6 by protein arginine *N*-methyltransferase 1

Masako Inamitsu[a], Susumu Itoh[a,*], Ulf Hellman[b], Peter ten Dijke[c], Mitsuyasu Kato[a]

[a] *Department of Experimental Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan*
[b] *Ludwig Institute for Cancer Research, Biomedical Center Box 595, Uppsala University, 751 24 Uppsala, Sweden*
[c] *Department of Molecular Cell Biology, Leiden University Medical Center, Postzone S-1-P, Postbus 9600, 2300 RC, Leiden, The Netherlands*

Received 19 August 2006; revised 14 October 2006; accepted 8 November 2006

Available online 14 November 2006

Edited by Veli-Pekka Lehto

Abstract **Signal transduction pathways utilize posttranslational modifications to regulate the activity of their components in a temporal-spatial and efficient fashion. Arginine methylation is one of the posttranslational modifications that can result in monomethylated-, asymmetric dimethylated- and/or symmetric dimethylated-arginine residues in proteins. Here we demonstrate that inhibitory-Smads (Smad6 and Smad7), but not receptor-regulated- (R-)Smads and the common-partner Smad4, can be methylated by protein arginine *N*-methyltransferase (PRMT)1. Using mass-spectrometric analysis, we found that PRMT1 dimethylates arginine^{74} (Arg^{74}) in mouse Smad6. PRMT1 interacts with the N-terminal domain of Smad6 in which Arg^{74} residue is located. Assays examined so far have shown no significant differences between the functions of Smad6 and those of methylation-defective Smad6 (Smad6R74A). Both wild-type and Smad6R74A were equally efficient in blocking BMP-induced growth arrest upon their ectopic expression in HS-72 mouse B-cell hybridoma cells.**

Keywords: BMP; I-Smad; PRMT1; Smad6; Smad7

1. Introduction

Posttranslational modification of proteins, such as phosphorylation, acetylation, glycosylation, ubiquitination and SUMOylation, allows for structural and functional diversity, and is a mechanism frequently used to regulate cellular signaling events. Although methylation of arginine residues was discovered more than 30 years ago [1], the functional significance of methylated arginine has been demonstrated only recently. To date, nine protein arginine methyltransferases (PRMTs) have been isolated and classified into two groups: type I and type II enzymes [2,3]. While both types can catalyze ω-N^G-monomethylation of arginine as an intermediate, type I enzymes (PRMT1, PRMT3, PRMT4, PRMT6 and PRMT8) promote asymmetric ω-N^G,N^G-dimethylation of arginine residue, whereas type II enzymes (PRMT5, PRMT7 and PRMT9) catalyze the formation of symmetric ω-N^G,N'^G-dimethylarginine. No enzymatic activity has been documented for PRMT2 in spite of its structural similarity to other PRMT family members [2,3]. PRMT1-deficient mice die at E6.5, whereas PRMT4-deficient mice can survive till late embryogenesis or perinatally. Thus, the lack of these PRMTs cannot be compensated by other PRMTs [4,5]. Many of the proteins targeted by PRMTs possess glycine–arginine rich (GAR) domains. In a GAR domain, RGG, RG repeats or RXR are sequence motifs used by PRMTs to methylate an arginine residue in proteins [6]. Arginine methylation by PRMTs regulates subcellular localization of proteins, enzymatic activities and protein–protein interactions [7]. The methylation status of Sam68 RNA binding protein determines its subcellular localization [8]. Exonuclease activity of MRE11 is abrogated by substitution of an arginine residue regularly methylated by PRMT1 [9]. Furthermore, certain transcription factors or their co-activators can be arginine-methylated by PRMTs, affecting their transcriptional activity [10–13].

Transforming growth factor-β (TGF-β) is a multi-functional cytokine that regulates cell division, differentiation, migration, adhesion, organization and death [14]. The TGF-β superfamily is classified into three subfamilies: TGF-βs, activins, and bone morphogenetic proteins (BMPs). Perturbation of TGF-β signaling has been implicated in oncogenesis, fibrosis, immune disorders and vascular disorders [15]. Signaling of TGF-β family members occurs via the formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors after ligand binding to receptors [16]. The type I receptor, termed activin receptor-like kinase (ALK), is a direct substrate for the constitutively active type II receptor kinase. Ligand binding induces the assembly between type I and type II receptors with ligand and promotes phosphorylation of the GS domain in the type I receptor by active type II receptor kinase, followed by activation of type I receptor kinase. Subsequently, active type I receptor kinase directly phosphorylates receptor-regulated (R)-Smads, resulting in the propagation of the signal in the cells. Phosphorylated R-Smads can make a complex with the common-partner Smad (Co-Smad), Smad4, to form ternary complexes, which accumulate in the nucleus, where they regulate the transcription of target genes [17,18].

The inhibitory Smads (I-Smads), Smad6 and Smad7, form a distinct subfamily of Smads that block TGF-β family signaling. Smad6 preferentially antagonizes BMP signaling, whereas Smad7 is a general inhibitor for TGF-β family signaling. I-Smads stably associate with the activated type I receptor to prevent R-Smads from being phosphorylated by type I receptor kinase. Recently, it has been reported that I-Smads recruit E3 ubiquitin ligases to the activated type I receptor and mediate proteasome-dependent degradation of the type I receptor. Consequently, TGF-β signaling is terminated [17]. In addition, Smad7 recruits both GADD34 and the catalytic subunit of

*Corresponding author. Fax: +81 29 853 3944.
E-mail address: sitoh@md.tsukuba.ac.jp (S. Itoh).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.11.008

protein phosphatase 1 to the activated type I receptor, resulting in promotion of dephosphorylation and inactivation of the activated type I receptor [19]. Moreover, it is known that Smad6 acts together with Hoxc-8, CtBP or HDACs as a transcriptional co-repressor [20].

I-Smads have been found to receive posttranslational modifications such as ubiquitination, acetylation and phosphorylation [21–23]. The balance of acetylation, deacetylation and ubiquitination of lysine residues in Smad7 is critical for its stability [24]. It was recently reported that phosphorylation of Smad6 by protein kinase X is required for differentiation of HL-60 cells [25]. Of the Smad family, our study showed that PRMT1 specifically methylates I-Smads. Moreover, we identified the methylated arginine residue in Smad6.

2. Materials and methods

2.1. Expression plasmids

Mouse Smad6R74A mutant was made using a QuickChange site-directed mutagenesis kit (Stratagene) with pcDNA3-Flag-mouse Smad6 [26] as the template. Constitutively active ALK6 (ALK6ca)/V5 was constructed by ligation of the insert from pcDNA3-ALK6ca/HA [27] with pcDNA3.1-V5-His-A (Invitrogen). Subsequently, the insert was ligated into the pcDEF3 vector again [28]. GST-PRMT1, GST-PRMT4, GST-PRMT5, GST-PRMT6, GST-GAR, HA-PRMT1, Flag-Smurf1ca and Myc-CtBP were kindly provided by Drs. Stallcup, Bedford, Pestka, Imamura and Feng [29–33]. The constructs for pcDNA3-Flag-Smad6, pcDNA3-Flag-Smad6N, pcDNA3-Flag-Smad6C, pcDNA3-Myc-Smad6, pcDNA3-Flag-Smad7, GST-Smad3, GST-Smad4 and $(SBE)_4$-luc have previously been described [27,34–38]. pGEX-4T-1 (GST alone) and CH110 for normalization of luciferase activity were purchased from Amersham Biosciences. GST-Smad5, GST-Smad6, GST-Smad6R74A, GST-Smad6(R15A,R17A), GST-Smad6(R74A,R82A), GST-Smad6R383A and GST-Smad7 were generated using pGEX-4T-1.

2.2. Cell culture and transfections

COS7, HepG2 and HS-72 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal calf serum (FCS, Bio-west) and 1×MEM non-essential amino acids (NEAA) (Sigma). For selection of stable transformants with pcDNA3-Flag-Smad6R74A in HS-72 cells, the cells were cultured in the presence of 500 μg/ml G418 (Gibco). All media included penicillin and streptomycin as antibiotics. The HS-72 transformant expressing Flag-Smad6, termed Sma6A, was obtained from Dr. Ishisaki [39].

2.3. Immunoprecipitation and Western blotting

To detect methylated Smads, indicated plasmids were transfected in COS7 cells at 5×10^5 cells/6 cm-dish using FuGENE 6 (Roche). Forty hours after transfection, the cells were lysed in 0.5 ml of TNE buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 5 μg/ml leupeptin, 100 U/ml aprotinin, 2 mM sodium vanadate, 40 mM NaF and 20 mM β-glycerophosphate). After being precleared with protein G-Sepharose beads (Amersham Biosciences) for 30 min at 4 °C, the cell lysates were incubated with anti-Flag M5 antibody (Sigma) for 2 h at 4 °C. Subsequently, protein G-Sepharose beads were added to the reaction mixture and incubated for 30 min at 4 °C. After the immunoprecipitates were washed three times with TNE buffer, the proteins in the immunoprecipitates and the aliquots of total cell lysates were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a Hybond-C Extra membrane (Amersham Biosciences). The membrane was subsequently probed with anti-monomethyl- and dimethyl-arginine antibody (Abcam). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham Biosciences) and chemiluminescent substrate (Pierce). The expression of proteins in total cell lysates was detected by Western blotting using anti-Flag M5 or anti-HA12CA5 (Roche) antibody. The detection of the interactions between Flag-Smad6 and HA-PRMT1 was performed by immunoprecipitation followed by Western blotting according to the above method except that anti-HA antibody was used in immunoprecipitation and anti-Flag M5 antibody was used in Western blotting.

2.4. In vitro methylation assay

All of the GST fusion proteins were purified using GSH-Sepharose 4B (Amersham Biosciences). GST-Smads (5 μg) were reacted with 2 μg of GST-PRMT1 plus 4 μl of *S*-adenosyl-L-[methyl-^{3}H] methionine ([^{3}H]AdoMet; 37 MBq/ml, Amersham Biosciences) in 50 μl of PBS for 90 min at 37 °C. Subsequently, samples were loaded on 7.5% SDS–PAGE and stained with 0.05% Coomassie Brilliant Blue R-250 (CBB), followed by detection of [^{3}H]-labeled proteins using BAS2500 (Fuji film).

2.5. MALDI-ToF MS analysis

GST-Smad6 or GST-Smad6R74A was incubated with GST-PRMT1 and 1.67 mM AdoMet in PBS for 90 min at 37 °C. Each sample was mixed with 2× sample buffer (4% SDS, 20 mM dithiothreitol, 20% glycerol, 125 mM Tris and 0.002% bromophenol blue) and boiled at 98 °C for 10 min, and then reacted with 20 mM iodoacetamide at 25 °C for 20 min in a dark room. Samples were then applied on 7.5% SDS–PAGE and stained with CBB. Corresponding bands for GST-Smad6 or its mutant were excised from the gel and treated for in-gel trypsin digestion as previously described [40]. Briefly, the gel bands were washed with acetonitrile and ammonium bicarbonate. After CBB was de-stained, the gels were dried with neat acetonitrile. A solution of modified sequence grade porcine trypsin (Promega) was added and allowed to soak into the gel pieces on ice. After incubation overnight at 30 °C and acidification, the digests were analyzed for mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) on a Bruker Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany). The instrument settings were those optimized for analytes up to about 4000 Da, and the matrix used was α-cyano 4-hydroxy-cinnamic acid. The search for peptides representing methylated arginine residues was performed using GPMAW (Lighthouse Data, Odense, Denmark). The sequence coverage for GST-Smad6 in the presence of PRMT1 was 39%, while that for GST-Smad6 alone was 42%.

2.6. Detection of phosphorylated Smad5

HS-72 stable transformants were cultured without FCS 12 h before stimulation with 500 ng/ml BMP-6, which was kindly provided by Dr. Sampath. One hour after stimulation with BMP-6, the cells were lysed and loaded on 8% SDS–PAGE. Anti-phosphorylated Smad1/5 (pS1) [41], anti-Smad5 [42] and anti-Flag M5 antibodies were used as primary antibodies. The detection method was the same as that described above.

2.7. MTT assay

To observe the growth inhibition of HS-72 stable transformants by BMP-6, MTT assay was performed [43].

3. Results

3.1. Methylation of I-Smads by PRMT1

The PRMT family can catalyze arginine methylation of a number of transcriptional factors [7]. To examine if Smads are substrates for the PRMT family, we examined whether GST-Smads can be methylated by GST-PRMTs *in vitro*. As seen in Fig. 1a, GST-Smad6 and GST-Smad7 were efficiently methylated by GST-PRMT1, whereas GST-Smad3 and GST-Smad5 (termed TGF-β/activin and BMP R-Smads, respectively), as well as GST-Smad4 and GST alone were not, or only very weakly (Fig. 1a and c). In addition, GST-PRMT4, GST-PRMT5 and GST-PRMT6 did not catalyze methylation of GST-Smads (data not shown). To confirm that PRMT1 can catalyze methylation of I-Smads *in vivo*, we transfected I-Smads with or without PRMT1 in COS7 cells. Then, methylated I-Smads after immunoprecipitation using Flag antibody were detected with anti-methylarginine antibody. Both Smad6

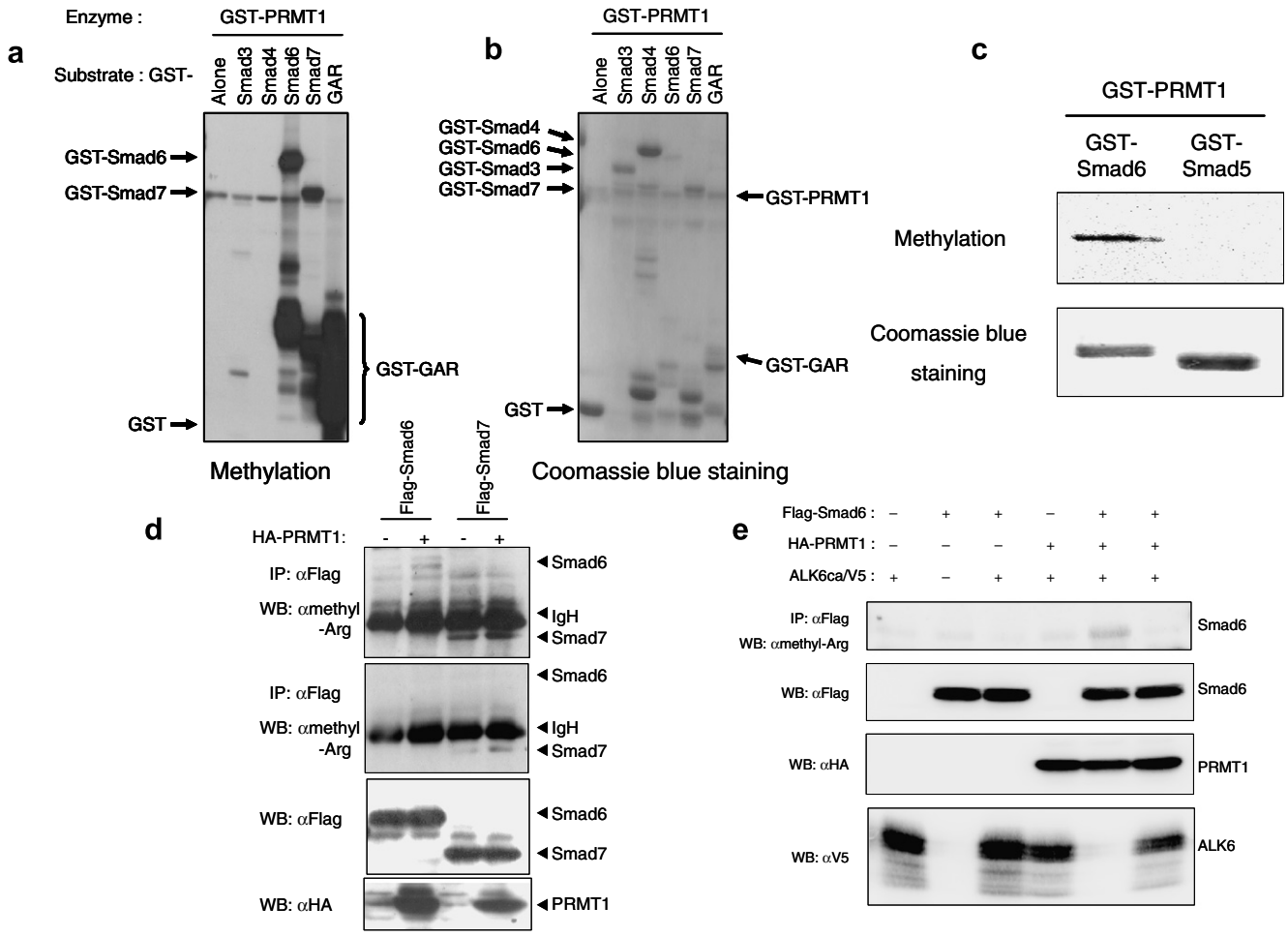

Fig. 1. I-Smads are substrates for PRMT1. (a, b) *In vitro* methylation of Smad proteins by PRMT1. GST-PRMT1 was incubated with GST-Smad3, GST-Smad4, GST-Smad6, GST-Smad7 or GST alone in the presence of [^{3}H]AdoMet as a methyl donor. GST-GAR was used as a positive control for methyltransferase activity. (a) [^{3}H]-labeled proteins visualized by autoradiography and (b) total proteins visualized by Coomassie blue staining. (c) Smad5 is not methylated by PRMT1. GST-PRMT1 was incubated with GST-Smad5 or GST-Smad6 in the presence of [^{3}H]AdoMet. Proteins were visualized by autoradiography (upper panel) or Coomassie blue staining (lower panel). (d) PRMT1 catalyzes methylation of I-Smads in COS7 cells. Flag-Smad6 and Flag-Smad7 were transfected in COS7 cells with or without HA-PRMT1. To detect methylated Smads, cell lysates were immunoprecipitated with anti-Flag M5 antibody, followed by Western blotting with anti-methyl-arginine antibody (upper and second panels). Although the intensity of the band corresponding to methylated Smad6 was very weak, the membrane shown in the upper panel was exposed for a longer time than that shown in the second panel. Using total cell lysates, expression controls for Smads (third panel) and PRMT1 (lower panel) were shown by anti-Flag M5 and anti-HA12CA5 antibodies, respectively. (e) Methylation of Smad6 decreases upon ALK6 activation in COS7 cells. Flag-Smad6 was transfected in COS7 cells in combination with HA-PRMT1 and ALK6ca/V5. To detect methylated Smad6, cell lysates were immunoprecipitated with anti-Flag M5 antibody, followed by Western blotting with anti-methyl-arginine antibody (upper panel). Using total cell lysates, expression controls for Smad6 (second panel), PRMT1 (third panel) and ALK6 (lower panel) were shown by anti-Flag M5, anti-HA12CA5 and anti-V5 antibodies, respectively.

and Smad7 were methylated when PRMT1 was co-transfected although the level of methylation in Smad7 was higher than that in Smad6 (Fig. 1d). Next, we examined if stimulation of cells with BMP influences methylation of Smad6 by PRMT1. As seen in Fig. 1e, a constitutively active BMP type I receptor termed ALK6ca, which transduces BMP signaling without ligand stimulation, significantly reduced PRMT1-mediated Smad6 methylation.

3.2. Identification of methylated arginine residue in Smad6

To identify methylated arginine residue(s) in I-Smads, we analyzed trypsinized peptides from I-Smads whose methylation was catalyzed by PRMT1 *in vitro* using MALDI-ToF MS. We identified five possible candidates for a methylated arginine residue in Smad6. Due to the high background, we could not succeed in identifying methylated arginine residues in Smad7 (data not shown). Thus, we focused on methylation of Smad6 in the subsequent experiments. As an initial step, five arginine residues that are possibly methylated by PRMT1 were substituted with alanine residues. We then investigated *in vitro* methylation of each GST-Smad6 mutant by GST-PRMT1. Of the GST-Smad6 mutants, GST-Smad6(R74A,R82A) was not methylated (Fig. 2a). Subsequently, we found that substitution of Arg^{74} to Ala in Smad6 blocks methylation of Smad6 by PRMT1 (Fig. 2b and c). It has been reported that PRMT1 can asymmetrically dimethylate and/or monomethylate an arginine residue in proteins [7]. To further confirm how Arg^{74} in Smad6 is modified by PRMT1, MALDI-ToF MS analysis was performed using GST-Smad6 or GST-Smad6R74A in the absence or presence of GST-PRMT1. By comparing the mass

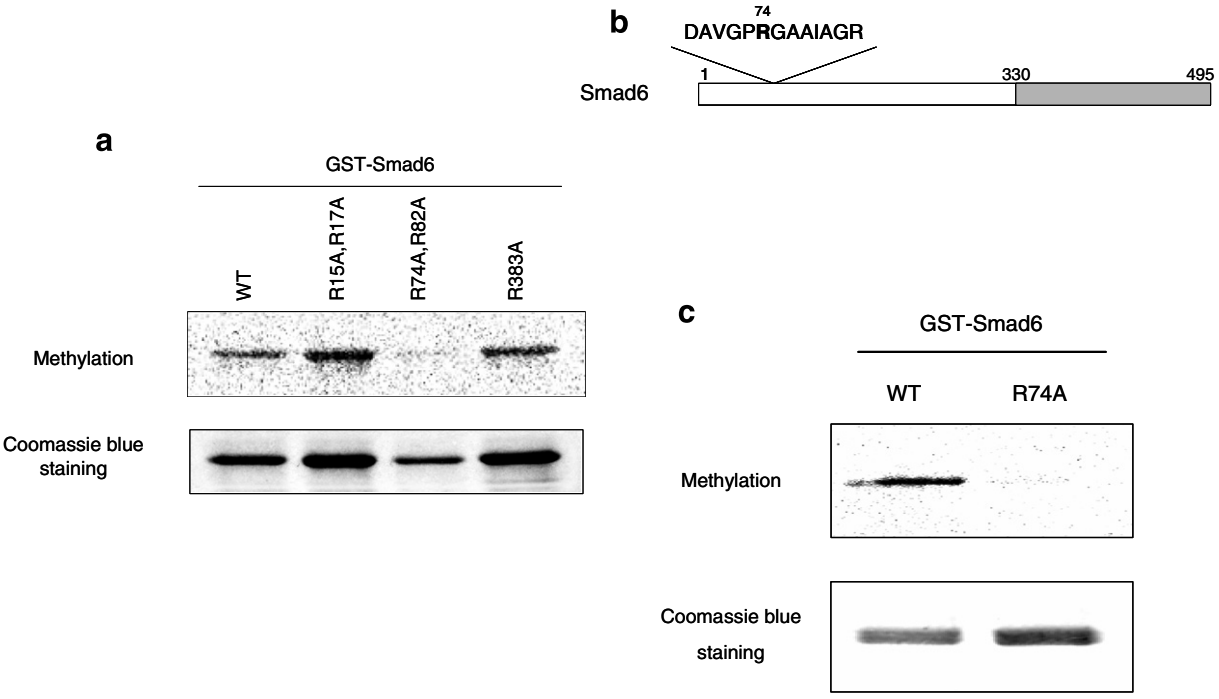

Fig. 2. Arg^{74} in mouse Smad6 is a target for methylation by PRMT1. (a) Methylation of Smad6 (R74A,R82A) by PRMT1 is drastically decreased *in vitro*. GST-PRMT1 was incubated with GST-Smad6 or its mutants in the presence of [^{3}H]AdoMet. Proteins were visualized by autoradiography (upper panel) or Coomassie blue staining (lower panel). (b) PRMT1-methylated arginine residue in mouse Smad6. The position of the methylated arginine in Smad6 is indicated in bold. The grey box indicates the MH2 domain. (c) Smad6R74A is not methylated by PRMT1 *in vitro*. GST-PRMT1 was incubated with GST-Smad6 or GST-Smad6R74A in the presence of [^{3}H]AdoMet. Proteins were visualized by autoradiography (upper panel) or Coomassie blue staining (lower panel). (d) Analysis of methylated peptides in Smad6 by MALDI-ToF-MS. GST-Smad6 or GST-Smad6R74A was incubated with AdoMet *in vitro* in the absence or presence of GST-PRMT1. The proteins were resolved by SDS–PAGE and then stained with Coomassie blue. Each apparent band at 65 kDa was excised, in-gel digested with trypsin, and analyzed by MALDI-ToF-MS. The arrow indicated the peak corresponding to the peptide including a dimethylarginine residue. (e) Smad6R74A is not methylated in COS7 cells. Flag-Smad6 or Flag-Smad6R74A was transfected in COS7 cells in the absence or presence of HA-PRMT1. To detect methylated Smad6, cell lysates were immunoprecipitated with anti-Flag M5 antibody, followed by Western blotting with anti-methyl-arginine antibody (upper panel). Using total cell lysates, expression controls for Smad6 (middle panel) and PRMT1 (lower panel) were shown with anti-Flag M5 and anti-HA12CA5 antibodies, respectively.

fingerprints of the two samples, we detected the methylated sample, which has a unique mass of 1238.67 Da, representing the peptide DAVGPR(2Me) GAAIAGR. The corresponding peptide in the mutated variant DAVGPAGAAIAGR was demonstrated by the mass 1125.60, and this peak was not affected by incubation with GST-PRMT1. Obviously, this mass was not found in the methylated protein. Thus, our data showed that Arg^{74} in Smad6 was dimethylated *in vitro* (Fig. 2d). To parallel the *in vitro* methylation assay, COS7 cells were transfected with Flag-Smad6 or Flag-Smad6R74A with or without HA-PRMT1. Subsequently, methylated Smad6 was detected with an anti-methyl-arginine antibody. Fig. 2e shows that Smad6, but not Smad6R74A, was methylated by PRMT1, indicating that Arg^{74} in Smad6 is a predominant site for methylation both *in vivo* and *in vitro*. However, it is possible that substitution of Arg^{74} to Ala in Smad6 affects the ternary structure of Smad6 to interfere access of PRMT1 to other arginine residue(s) in Smad6 or methylation of Arg^{74} is required for methylation of other arginine residue(s) in Smad6. Currently, therefore, the presence of one or more additional methylation sites in Smad6 cannot be ruled out.

3.3. Interaction of Smad6 with PRMT1

Since PRMT1 methylates Smad6, both proteins should interact with each other. To examine this possibility, Smad6 or Smad6R74A was transfected in COS7 cells with HA-PRMT1 in the absence or presence of ALK6ca. As seen in Fig. 3a, PRMT1 could associate with Smad6 as well as Smad6R74A, although the binding between PRMT1 and Smad6 was marginally stronger than that between PRMT1 and Smad6R74A. In addition, ALK6ca had no significant effect on the interaction between Smad6 and PRMT1. To determine the region of Smad6 that interacts with PRMT1, Flag-tagged Smad6N and Flag-tagged Smad6C were transfected with HA-tagged PRMT1 in COS7 cells, and this was followed by immunoprecipitation with Flag antibody and Western blotting with HA antibody. Fig. 3b shows that Smad6 lacking the MH2 domain, Smad6N, binds to PRMT1, but Smad6C does not.

3.4. Functional differences between Smad6 and Smad6R74A

Smad6 is known to inhibit BMP signaling (26, 39). To examine if, like Smad6, Smad6R74A has an ability to inhibit BMP signaling, $(SBE)_4$-luc, whose activity is induced by BMP (38), was co-transfected with Smad6 or Smad6R74A in HepG2 cells. Then, the cells were stimulated with BMP. As seen in Fig. 4a, not only Smad6R74A but also Smad6 can efficiently block BMP-induced luciferase activity in a dose-dependent manner. We also tried to explore interaction of Smad6R74A with ALK6ca (26), Smurf1 (32) or CtBP (33) because Smad6

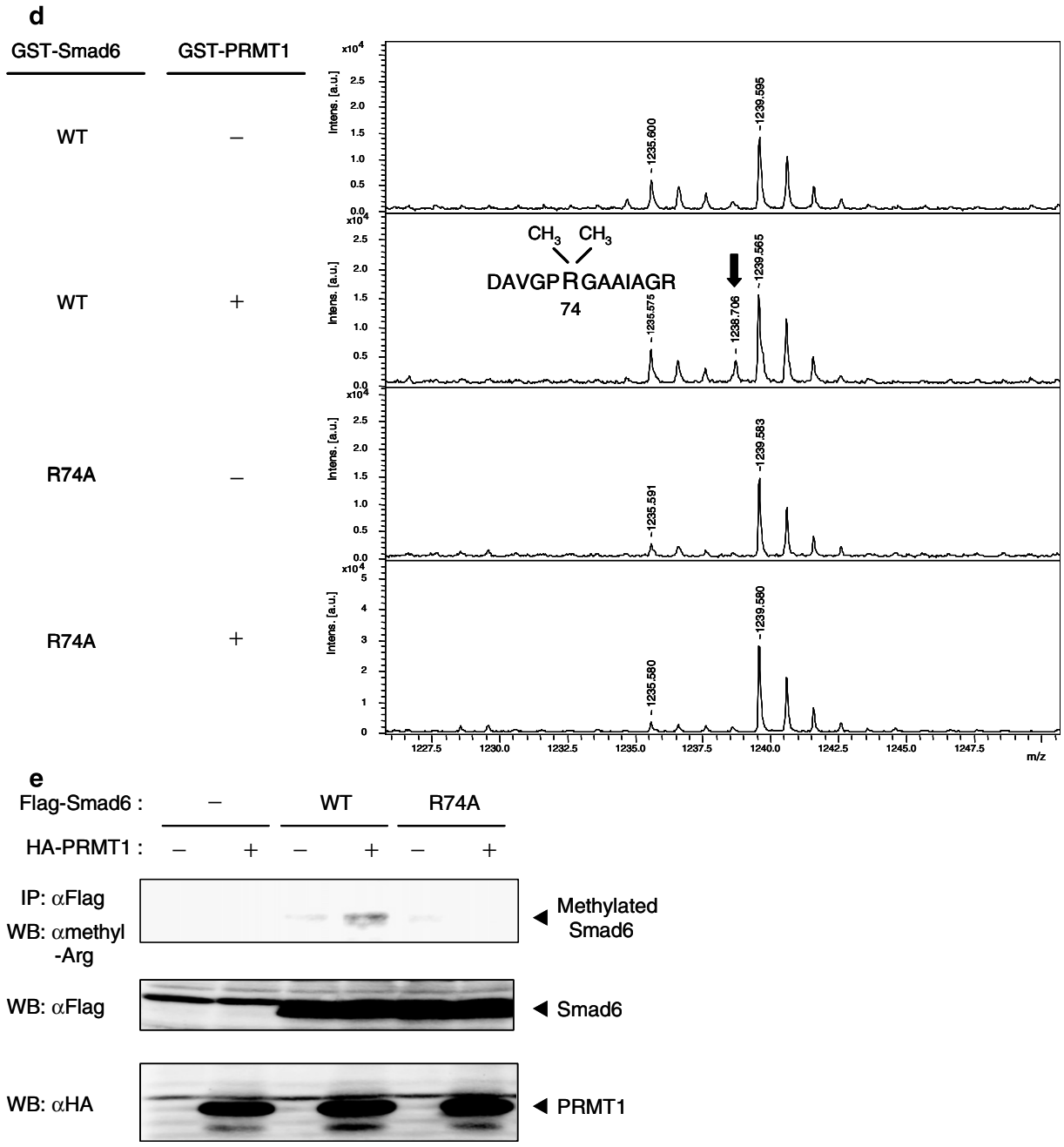

Fig. 2 (*continued*)

mediates its effect via interaction with these proteins. However, we could not observe any differences between Smad6 and Smad6R74A on interaction with ALK6ca, Smurf1 or CtBP although both the wild-type and mutant Smad6 could interact with them (Fig. 4b–d).

Since Smad6 inhibits BMP-induced growth inhibition and apoptosis of HS-72 mouse B-cell hybridoma cells [39], we examined the effect of ectopic expression of Smad6R74A or wild-type Smad6 on BMP-induced response. A Flag-Smad6R74A expression vector with a G418-resistant marker was stably transfected into HS-72 cells, and two transformants, Smad6R74A-17 and Smad6R74A-22, that express Smad6R74A at a high level were obtained (data not shown). In the subsequent experiments, the properties of these two clones were compared with those of Sma6A expressing wild-type Smad6 [39]. Using a specific antibody for phosphorylated BMP R-Smad (termed pS1), we first compared the phosphorylation of endogenous BMP R-Smads induced by BMP between parental HS-72 cells and transformants for Smad6 or its mutants. Phosphorylation of BMP R-Smads by BMP was almost abolished by both Smad6 and its mutants (Fig. 5a). To examine if Smad6 mutants affect BMP-induced growth arrest in HS-72 cells, we carried out MTT assay using Sma6A, Smad6R74A-17 and Smad6-R74A-22. Consistent with the inhibition of BMP R-Smad

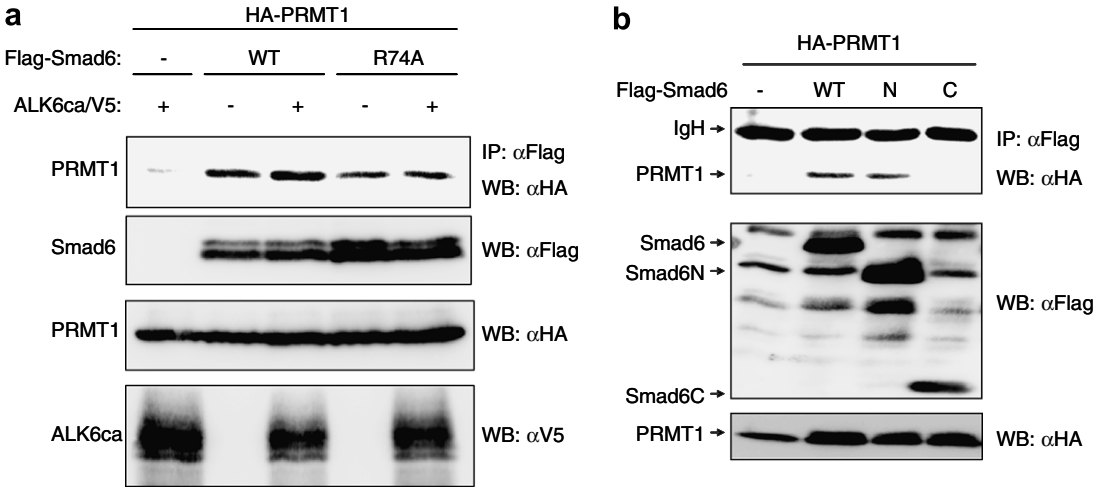

Fig. 3. Interaction of Smad6 with PRMT1. (a) Flag-Smad6 and Flag-Smad6R74A were transfected in COS7 cells with HA-PRMT1 in the absence or presence of ALK6ca/V5. Cell lysates were immunoprecipitated with anti-Flag M5 antibody, followed by Western blotting with anti-HA12CA5 antibody (upper panel). Using total cell lysates, expression controls for Smad6 (second panel), PRMT1 (third panel) and ALK6ca (lower panel) were shown with anti-Flag M5, anti-HA12CA5 and anti-V5 antibodies, respectively. (b) Smad6 lacking the MH2 domain can bind to PRMT1. Flag-Smad6 and its mutants were expressed together with HA-PRMT1 in COS7 cells. Proteins coprecipitated with Smad6 were detected using anti-HA12CA5 antibody. The expression of Smad6 or PRMT1 was observed with anti-Flag M5 (middle panel) or anti-HA12CA5 antibody (lower panel).

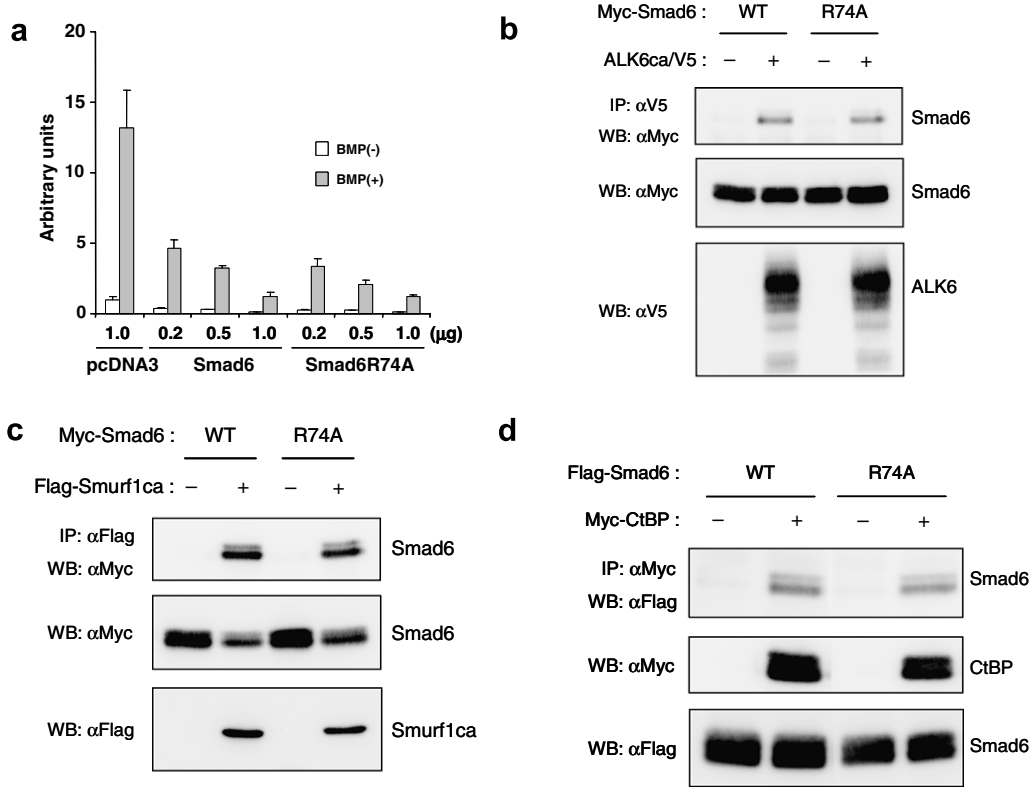

Fig. 4. Effect of Smad6R74A on known functions of Smad6. (a) Effect of Smad6R74A on BMP-induced reporter activity. Different doses of Smad6 or Smad6R74A were transfected with $(SBE)_4$-luc in HepG2 cells with or without 25 ng/ml BMP-6 for 18 h. Luciferase values were normalized for transfection efficiency. All values represent means ± S.D. ($n = 3$). (b) Interaction of Smad6 or its mutant with active BMP type I receptor. Myc-Smad6 or Myc-Smad6R74A was transfected in COS7 cells in the absence or presence of ALK6ca/V5. Cell lysates were immunoprecipitated with anti-V5 antibody, followed by Western blotting with anti-Myc antibody (upper panel). Using total cell lysates, expression controls for Smad6 (middle panel) and ALK6ca (lower panel) were shown with anti-Myc and anti-V5 antibodies, respectively. (c) Smad6R74A has the ability to bind to Smurf1 similar to Smad6. COS7 cells were transfected with Myc-Smad6 or Myc-Smad6R74A in the presence or absence of Flag-Smurf1ca, which is a mutant deficient of E3 ligase activity. Immunoprecipitation was performed with anti-Flag M5 antibody. Subsequently, proteins coprecipitated with Smad6 were detected with anti-Myc antibody (upper panel). Using total cell lysates, expression controls for Smad6 (middle panel) and Smurf1 (lower panel) were shown with anti-Myc and anti-Flag M5 antibodies, respectively. (d) Interaction of CtBP with Smad6 or Smad6R74A. Myc-CtBP was transfected with either Flag-Smad6 or Flag-Smad6R74A in COS7 cells. After immunoprecipitation with anti-Myc antibody, Western blotting was performed with anti-Flag M5 antibody (upper panel). Using total cell lysates, expression controls for CtBP (middle panel) and Smad6 (lower panel) were shown with anti-Myc and anti-Flag M5 antibodies, respectively.

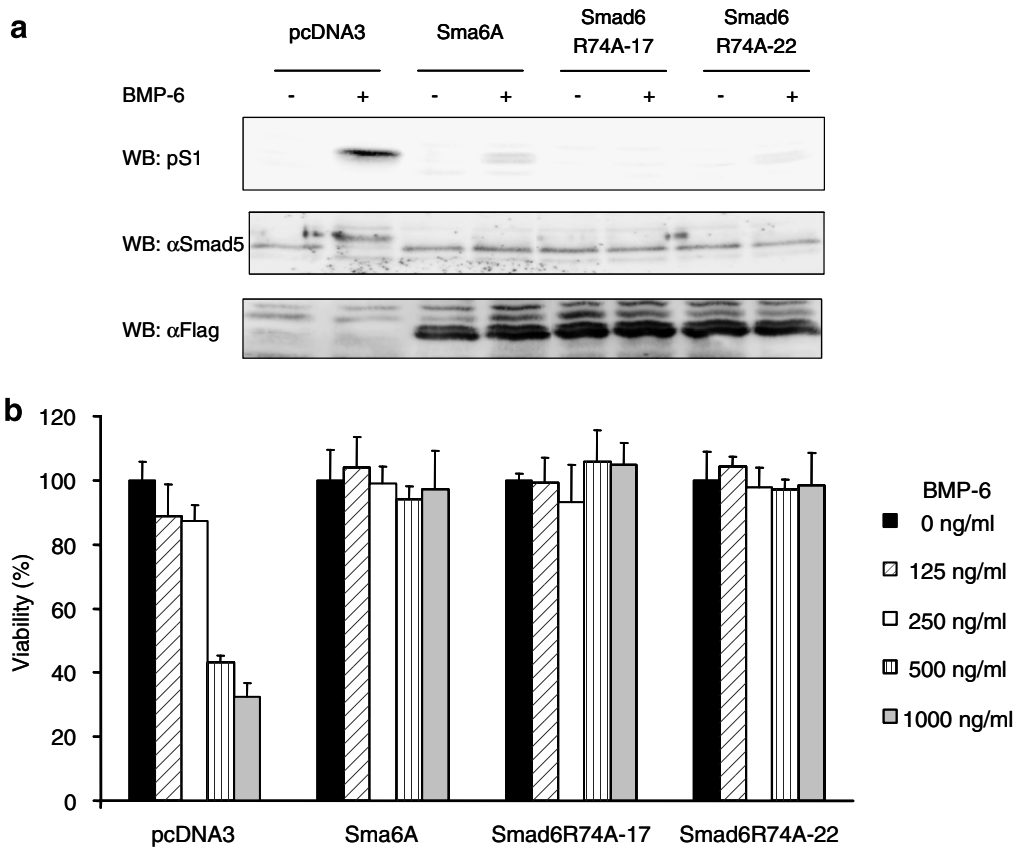

Fig. 5. Effect of Smad6R74A on BMP-mediated Smad1/5 phosphorylation and growth arrest in HS-72 cells. (a) C-terminal phosphorylation of Smad1/5 was investigated in HS-72 cells stably expressing Flag-Smad6 (Sma6A) or Flag-Smad6R74A (two independent clones; R74A-17 and R74A-22). After starvation, cells (1×10^6 cells) were treated with 500 ng/ml BMP-6 for 1 h. The expression of phosphorylated Smad1/5, Smad5 and Smad6 in total cell lysates were detected with pS1, anti-Smad5 and anti-Flag M5 antibodies, respectively. C-terminal phosphorylated Smad1/5 revealed slower migration in SDS–PAGE than did Smad1/5. (b) The cells were cultured with various concentrations of BMP-6 for 48 h and their viabilities were monitored by MTT assay. The percent viability was calculated by the following formula: percent viability = $100 \times (A_{584-630\ nm}$ with BMP-6/ $A_{584-630\ nm}$ without BMP-6). Data are expressed as the means ± S.D. of triplicate cultures.

phosphorylation, BMP-induced growth arrest was completely blocked in both the Smad6R74A-expressing cells and Sma6A (Fig. 5b).

4. Discussion

Posttranslational modification plays a prominent role in the regulation of Smad activity. Although phosphorylation, acetylation, glycosylation, ubiquitination and SUMOylation have been demonstrated in Smad proteins [17], here we report for the first time methylation of arginine residue(s) in Smads. Our results showed that I-Smads, Smad6 and Smad7, were methylated by PRMT1 *in vitro* and *in vivo*. Specificity is shown by the fact that R-Smads and Smad4 are not methylated on arginine residues, and that PRMT4, PRMT5 and PRMT6 show no significant activity for Smad targets. Arginine targeted by PRMT1, consisting of the RG motif, is conserved between human and mouse Smad6s (Arg^{75} for human Smad6, Arg^{74} for mouse Smad6), as well as between mouse Smad6 and Smad7 (Arg^{38} for mouse and human Smad7s). In the present study, we could not identify any methylated arginine residue in Smad7 that was dependent on the PRMT1 treatment. We are investigating the possibility that the conserved arginine residue in Smad7 is methylated by PRMT. The interaction between PRMT1 and Smad6 was demonstrated with the co-immunoprecipitation assay. Since the N-terminal part of Smad6 interacts with PRMT1, the region around Arg^{74} residue might be important for Smad6 to interact with PRMT1.

Both wild-type and methylation-defective mutant Smad6R74A were equally efficient in blocking BMP-induced growth inhibition upon their ectopic expression in HS-72 mouse hybridoma cells. Smad6 is known to mediate its effects via interaction with other proteins [20,23,26,32,33,43–46]. However, comparison of the interaction of wild-type Smad6 and its methylation-defective mutant with the activated BMP type I receptors, Smurfs, arkadia, phosphorylated Smad1, TAK1, TAB1, CtBP, AMSH and HDAC1 revealed no significant differences (Fig. 4 and data not shown). We are currently investigating the functional significance of methylation using more unbiased approaches such as transcriptional profiling of cells that ectopically express Smad6R74A mutant or wild-type Smad6.

Acknowledgements: This research was supported by an AstraZeneca Research Grant 2004 (S.I.), the Kowa Life Science Foundation (S.I.), the Kato Memorial Bioscience Foundation (S.I.), a Grants-in-aid for Scientific Research (S.I. and M.K.), a Genome Network Project grant from the Ministry of Education, Culture, Sports, Science and

Technology (M.K.), the Dutch Cancer Society, and EC 6th framework STREP Tumor-Host Genomics (P.t.-D.). We thank Drs. Moustakas, Stallcup, Bedford, Pestka, Imamura, Ishisaki, Feng and Sampath for valuable reagents.

References

[1] Paik, W.K. and Kim, S. (1967) Enzymatic methylation of protein fractions from calf thymus nuclei. Biochem. Biophys. Res. Commun. 29, 14–20.
[2] Blanchet, F., Schurter, B.T. and Acuto, O. (2006) Protein arginine methylation in lymphocyte signaling. Curr. Opin. Immunol. 18, 321–328.
[3] Cook, J.R., Lee, J.-H., Yang, Z.-H., Krause, C.D., Herth, N., Hoffmann, R. and Pestka, S. (2006) FBXO11/PRMT9, a new protein arginine methyltransferase, symmetrically dimethylates arginine residues. Biochem. Biophys. Res. Commun. 342, 472–481.
[4] Pawlak, M.R., Scherer, C.A., Chen, J., Roshon, M.J. and Ruley, H.E. (2000) Arginine *N*-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable. Mol. Cell. Biol. 20, 4859–4869.
[5] Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M.C.-T., Aldaz, C.M. and Bedford, M.T. (2003) Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. Proc. Natl. Acad. Sci. USA 100, 6464–6468.
[6] McBride, A.E. and Silver, P.A. (2001) State of the arg: protein methylation at arginine comes of age. Cell 106, 5–8.
[7] Bedford, M.T. and Richard, S. (2005) Arginine methylation an emerging regulator of protein function. Mol. Cell 18, 263–272.
[8] Côté, J., Boisvert, F.-M., Boulanger, M.C., Bedford, M.T. and Richard, S. (2003) Sam68 RNA binding protein is an *in vivo* substrate for protein arginine *N*-methyltransferase 1. Mol. Biol. Cell 14, 274–287.
[9] Boisvert, F.-M., Déry, U., Masson, J.-Y. and Richard, S. (2005) Arginine methylation of MRE11 by PRMT1 is required for DNA damage checkpoint control. Genes Dev. 19, 671–676.
[10] Mowen, K.A., Schurter, B.T., Fathman, J.W., David, M. and Glimcher, L.H. (2004) Arginine methylation of NIP45 modulates cytokine gene expression in effector T lymphocytes. Mol. Cell 15, 559–571.
[11] Rezai-Zadeh, N., Zhang, X., Namour, F., Fejer, G., Wen, Y.-D., Yao, Y.-L., Gyory, I., Wright, K. and Seto, E. (2003) Targeted recruitment of a histone H4-specific methyltransferase by the transcription factor YY1. Genes Dev. 17, 1019–1029.
[12] Mowen, K.A., Tang, J., Zhu, W., Schurter, B.T., Shuai, K., Herschman, H.R. and David, M. (2001) Arginine methylation of STAT1 modulates IFNα/β-induced transcription. Cell 104, 731–741.
[13] Lee, D.Y., Teyssier, C., Strahl, B.D. and Stallcup, M.R. (2005) Role of protein methylation in regulation of transcription. Endocr. Rev. 26, 147–170.
[14] Roberts, A.B. and Sporn, M.B. (1990) The transforming growth factor-βs (Sporn, M.B. and Roberts, A.B., Eds.), Peptide Growth Factors and Their Receptors, Part I, Vol. 95, pp. 419–472, Springer-Verlag, Berlin.
[15] Massagué, J., Blain, S.W. and Lo, R.S. (2000) TGFβ signaling in growth control, cancer, and heritable disorders. Cell 103, 295–309.
[16] Heldin, C.-H., Miyazono, K. and ten Dijke, P. (1997) TGF-β signalling from cell membrane to nucleus through SMAD protein. Nature 390, 465–471.
[17] Massagué, J., Seoane, J. and Wotton, D. (2005) Smad transcription factors. Genes Dev. 19, 2783–2810.
[18] Shi, Y. and Massagué, J. (2003) Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113, 685–700.
[19] Shi, W., Sun, C., He, B., Xiong, W., Shi, X., Yao, D. and Cao, X. (2004) GADD34-PP1c recruited by Smad7 dephosphorylates TGFβ type I receptor. J. Cell Biol. 164, 291–300.
[20] Park, S.H. (2005) Fine tuning and cross-talking of TGF-β signal by inhibitory Smads. J. Biochem. Mol. Biol. 38, 9–16.
[21] Pulaski, L., Landström, M., Heldin, C.-H. and Souchelnytskyi, S. (2001) Phosphorylation of Smad7 at Ser-249 does not interfere with its inhibitory role in transforming growth factor-β-dependent signaling but affects Smad7-dependent transcriptional activation. J. Biol. Chem. 276, 14344–14349.
[22] Grönroos, E., Hellman, U., Heldin, C.-H. and Ericsson, J. (2002) Control of Smad7 stability by competition between acetylation and ubiquitination. Mol. Cell 10, 483–493.
[23] Koinuma, D., Shinozaki, M., Komuro, A., Goto, K., Saitoh, M., Hanyu, A., Ebina, M., Nukiwa, T., Miyazawa, K., Imamura, T. and Miyazono, K. (2003) Arkadia amplifies TGF-β superfamily signalling through degradation of Smad7. EMBO J. 22, 6458–6470.
[24] Simonsson, M., Heldin, C.-H., Ericsson, J. and Grönroos, E. (2005) The balance between acetylation and deacetylation controls Smad7 stability. J. Biol. Chem. 280, 21797–21803.
[25] Glesne, D. and Huberman, E. (2006) Smad6 is a protein kinase X phosphorylation substrate and is required for HL-60 cell differentiation. Oncogene 25, 4086–4098.
[26] Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M. and Miyazono, K. (1997) Smad6 inhibits signalling by the TGF-β superfamily. Nature 389, 622–626.
[27] Kawabata, M., Inoue, H., Hanyu, A., Imamura, T. and Miyazawa, K. (1998) Smad proteins exist as monomers *in vivo* and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors. EMBO J. 17, 4056–4065.
[28] Goldman, L.A., Cutrone, E.C., Kotenko, S.V., Krause, C.D. and Langer, J.A. (1996) Modifications of vectors pEF-BOS, pcDNA1 and pcDNA3 result in improved convenience and expression. Biotechniques 21, 1013–1015.
[29] Branscombe, T.L., Frankel, A., Lee, J.-H., Cook, J.R., Yang, Z., Pestka, S. and Clarke, S. (2001) PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. J. Biol. Chem. 276, 32971–32976.
[30] Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.-M., Schurter, B.T., Aswad, D.W. and Stallcup, M.R. (1999) Regulation of transcription by a protein methyltransferase. Science 284, 2174–2177.
[31] Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S. and Bedford, M.T. (2002) The novel human protein arginine *N*-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. J. Biol. Chem. 277, 3537–3543.
[32] Murakami, G., Watabe, T., Takaoka, K., Miyazono, K. and Imamura, T. (2003) Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads. Mol. Biol. Cell. 14, 2809–2817.
[33] Lin, X., Liang, Y.Y., Sun, B., Liang, M., Shi, Y., Brunicardi, F.C., Shi, Y. and Feng, X.-H. (2003) Smad6 recruits transcription corepressor CtBP to repress bone morphogenetic protein-induced transcription. Mol. Cell. Biol. 23, 9081–9093.
[34] Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S. and Gauthier, J.-M. (1998) Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J. 17, 3091–3100.
[35] Hanyu, A., Ishidou, Y., Ebisawa, T., Shimanuki, T., Imamura, T. and Miyazono, K. (2001) The N domain of Smad7 is essential for specific inhibition of transforming growth factor-β signaling. J. Cell Biol. 155, 1017–1027.
[36] Itoh, S., Ericsson, J., Nishikawa, J., Heldin, C.-H. and ten Dijke, P. (2000) The transcriptional co-activator P/CAF potentiates TGF-β/Smad signaling. Nucleic Acids Res. 28, 4291–4298.
[37] Itoh, S., Thorikay, M., Kowanetz, M., Moustakas, A., Itoh, F., Heldin, C.-H. and ten Dijke, P. (2003) Elucidation of Smad requirement in transforming growth factor-β type I receptor-induced responses. J. Biol. Chem. 278, 3751–3761.
[38] Jonk, L.J., Itoh, S., Heldin, C.-H., ten Dijke, P. and Kruijer, W. (1998) Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-β, activin, and bone morphogenetic protein-inducible enhancer. J. Biol. Chem. 273, 21145–21152.
[39] Ishisaki, A., Yamato, K., Hashimoto, S., Nakao, A., Tamaki, K., Nonaka, K., ten Dijke, P., Sugino, H. and Nishihara, T. (1999) Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells. J. Biol. Chem. 274, 13637–13642.

[40] Hellman, U. (2000) Sample preparation by SDS/PAGE and in-gel digestions in: Proteomics in Functional Genomics. Protein Structure Analysis (Jollès, P. and Jörnvall, H., Eds.), pp. 43–54, Birkhauser Verlag AG, Basel.
[41] Persson, U., Izumi, H., Souchelnytskyi, S., Itoh, S., Grimsby, S., Engström, U., Heldin, C.-H., Funa, K. and ten Dijke, P. (1998) The L45 loop in type I receptors for TGF-β family members is a critical determinant in specifying Smad isoform activation. FEBS Lett. 434, 83–87.
[42] Tamaki, K., Souchelnytskyi, S., Itoh, S., Nakao, A., Sampath, K., Heldin, C.-H. and ten Dijke, P. (1998) Intracellular signaling of osteogenic protein-1 through Smad5 activation. J. Cell. Physiol. 177, 355–363.
[43] Itoh, F., Asao, H., Sugamura, K., Heldin, C.-H., ten Dijke, P. and Itoh, S. (2001) Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. EMBO J. 20, 4132–4142.
[44] Hata, A., Lagna, G., Massagué, J. and Hemmati-Brivanlou, A. (1998) Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev. 12, 186–197.
[45] Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T. and Miyazono, K. (2001) Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. J. Biol. Chem. 276, 12477–12480.
[46] Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H. and Wrana, J.L. (2000) Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGFβ receptor for degradation. Mol. Cell 6, 1365–1375.